Small RNA and DNA from Plasma

User manual

NucleoSpin® miRNA Plasma

July 2014 / Rev. 03

MACHEREY-NAGEL
www.mn-net.com

Isolation of miRNA from plasma

Protocol-at-a-glance (Rev. 03)

		NucleoSpin® miRNA Plasma
1	**Prepare sample**	300 µL plasma or serum* 90 µL MLP Vortex 5 s RT, 3 min
2	**Precipitate protein**	30 µL MPP Vortex 5 s RT, 1 min 11,000 x *g*, 3 min
3	**Transfer supernatant**	Transfer clear supernatant to Collection Tube (2 mL, lid)
4	**Adjust binding conditions**	400 µL isopropanol Vortex 5 s
5	**Bind RNA and DNA**	Load sample on NucleoSpin® miRNA Column RT, 2 min 11,000 x *g*, 30 s
6	***Optional: Digest DNA***	***Optional:*** 1st 700 µL MW2 11,000 x *g*, 30 s 2nd 250 µL MW2 11,000 x *g*, 2 min 50 µL rDNase in Reaction Buffer for rDNase RT, 15 min
7	**Wash and dry**	1st 100 µL **MW1** 11,000 x *g*, 30 s 2nd 700 µL MW2 11,000 x *g*, 30 s 3rd 250 µL MW2 11,000 x *g*, 2 min
8	**Elute RNA**	30 µL RNase-free H_2O RT, 1 min 11,000 x *g*, 1 min

* Larger sample volumes can be processed when buffer volumes of MLP, MPP, and isopropanol are increased proportionally. Multiple loading is necessary in step 5 (see section 2.3 for more information).

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1 Components

1.1 Kit contents

	NucleoSpin® miRNA Plasma		
	10 preps	**50 preps**	**250 preps**
REF	**740981.10**	**740981.50**	**740981.250**
Lysis Buffer MLP	3 mL	13 mL	75 mL
Protein Precipitation Buffer MPP	5 mL	5 mL	25 mL
Reaction Buffer for rDNase	7 mL	7 mL	30 mL
rDNase, RNase-free (lyophilized)	1 vial (size C)	1 vial (size C)	5 vials (size C)
Wash Buffer MW1	10 mL	10 mL	35 mL
Wash Buffer MW2 (Concentrate)*	6 mL	25 mL	2 x 50 mL
RNase-free H_2O	13 mL	13 mL	13 mL
NucleoSpin® miRNA Columns (green rings)	10	50	250
Collection Tubes (1.5 mL)	10	50	250
Collection Tubes (2 mL)	10	50	250
Collection Tubes (2 mL, lid)	10	50	250
User manual	1	1	1

* For preparation of working solutions and storage conditions see section 3.

1.2 Reagents, consumables, and equipment to be supplied by user

Reagents

- 96–100 % ethanol
- Isopropanol

Consumables

- RNase-free disposable pipette tips

Equipment

- Manual pipettors
- Vortexer
- Centrifuge for microcentrifuge tubes
- Personal protection equipment (lab coat, gloves, goggles)

1.3 About this user manual

It is strongly recommended that first-time users of the **NucleoSpin® miRNA Plasma** kit read the detailed protocol section of this user manual. Experienced users, however, may refer to the Protocol-at-a-glance instead. The Protocol-at-a-glance is designed to be used only as a supplemental tool for quick referencing while performing the purification procedure.

All technical literature is available on the internet at ***www.mn-net.com***.

2 Product description

2.1 The basic principle

The **NucleoSpin® miRNA Plasma** kit offers the unique feature to isolate small RNA and DNA from plasma without the need to resort to the cumbersome phenol / chloroform extraction or a time consuming proteinase digest.

The sample material is denatured in Lysis Buffer MLP. The protein is then precipitated by Protein Precipitation Buffer MPP and pelleted by centrifugation.

After the removal of protein the binding conditions for nucleic acids are adjusted by adding isopropanol.

The small RNA and DNA are bound to the NucleoSpin® miRNA Column. Optionally, DNA can be removed by an on-column rDNase digest. The remaining nucleic acids are washed and eluted with minimal amounts of RNase-free water.

2.2 Kit specifications

- The **NucleoSpin® miRNA Plasma** kit is designed for the isolation of short RNA and DNA (< 1000 nt/bp) from cell free blood plasma or serum.
- **rDNase** is provided for an optional on-column digest to remove traces of genomic DNA.
- The eluted RNA and DNA are ready-to-use for all standard downstream applications, for example, qPCR, qRT-PCR, Northern Blot, chip hybridization.

Table 1: Kit specifications at a glance

Parameter	NucleoSpin® miRNA Plasma
Sample size	300 µL blood plasma or serum
Fragment size	< 1000 nt/bp
Binding capacity	200 µg
Elution volume	20–50 µL
Preparation time	40 min/10 preps (without rDNase digest) 70 min/10 preps (with rDNase digest)

2.3 Amount of starting material

The standard procedure allows to process 300 µL of sample material with only one loading step onto the NucleoSpin® miRNA Column. This is usually enough to detect also low abundance miRNA in plasma or serum.

If larger sample volumes are to be used to increase the sensitivity even further, the volumes of Buffer MLP and Buffer MPP as well as the isopropanol have to be increased proportionally. Multiple loading steps per sample are necessary.

But, consider that doubling or tripling the starting volume will result in an only 1–1.5 cycles earlier signal in qRT-PCR, which is rather insignificant for detection sensitivity compared to the much larger differences that occur from sample to sample or between different miRNAs. Furthermore, if plasma quality is low, co-purified RT-PCR inhibitors might require diluting the eluate and thereby counteract the increased yields.

Figure 1 shows qRT-PCR results from eight different plasma samples analyzed for miR-16 (very high expression) and miR-1 (very low expression). They differ in average by about 14 cycles, which is a difference in expression by 3–4 orders of magnitude (1.000–10.000 fold)! Doubling the amount of sample material would just shift the mean values from 32.6 to 31.6 (miR-1) and from 18.3 to 17.3 (miR-16). This is much less than the variation from sample to sample and can thus be neglected.

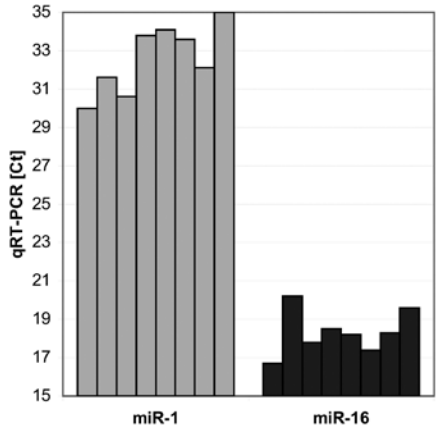


Figure 1: qRT-PCR quantification of miR-1 and miR-16
MiRNA from only 300 µL of eight different blood plasma samples were purified and eluted in 30 µL RNase-free water. 2 µL of each eluate were used for a 10 µL RT reaction (Applied Biosystems, TaqMan® MicroRNA RT Kit) with miRNA specific primers (Applied Biosystems, hsa-miR-1 MicroRNA Assay and hsa-miR-16 MicroRNA Assay). The RT reaction mix was diluted 1:10. Only 4 µL of the resulting 100 µL were used for the PCR reaction (Applied Biosystems TaqMan® Universal PCR Master Mix) in combination with the MicroRNA Assays for specific priming mentioned before.

2.3.1 Preparation of plasma from human EDTA blood

1. Centrifuge fresh blood sample for 10 min at 2,000 x *g.*
2. Remove the plasma without disturbing sedimented cells.
3. Freeze plasma at -20 °C for storage upon DNA isolation.
4. Thaw frozen plasma samples prior to DNA isolation and centrifuge for 3 min at ≥11,000 x *g* in order to remove residual cells, cell debris, and particulate matter. Use the supernatant for DNA isolation.

2.4 Proteinase K digest

A short protein digestion step might increase miRNA yield, especially for low quality, hemolyzed plasma. Add 10 µL of Proteinase K (~ 30 µg/µL in Buffer PB, see ordering information) to 300 µL plasma, incubate for 10 min at 37 °C, and then proceed with addition of Buffer MLP according to the protocol.

2.5 Addition of carrier

To improve RNA / DNA binding to the NucleoSpin® miRNA Column, carrier can be added to the sample after the removal of precipitated protein. Slightly higher yields could be found with 2 µg of glycogen or 5 µg of LPA (linear polyacrylamide). However, negligible effects were observed for Poly-A which furthermore interferes with a photometric quantification of the purified nucleic acids.

2.6 Elution procedures

The elution buffer volume does not only influence total yield and concentration of RNA and DNA, but does also influence the ratio between very small and larger oligonucleotides:

20 µL

The silica membrane is not completely wetted. Only weakly binding very small oligonucleotides like miRNA are eluted efficiently. Larger RNA and DNA are more likely to remain bound to the column. The eluted miRNA is highly concentrated.

30 µL (standard)

The standard elution buffer volume of 30 µL is sufficient to wet the silica membrane completely. It results a high total yield of miRNA / RNA / DNA and simultaneously maximizes the concentration.

50 µL

Increasing the elution buffer volume will further increase the final yield but consequently will reduce the concentration. The gain in yield will usually not compensate for the loss in sensitivity of miRNA detection caused by the dilution of the eluate. Furthermore, larger RNA and DNA will be eluted more efficiently, which might, however, be interesting for the analysis of circulating DNA.

3 Storage conditions and preparation of working solutions

Attention:

Buffers MLP and MW1 contain guanidinium thiocyanate. Wear gloves and goggles!

Storage conditions:

- Store lyophilized RNase-free rDNAse at +4 °C on arrival (stable for at least one year).
- All other kit components should be stored at room temperature (18–25 °C) and are stable for at least one year. Storage at lower temperatures may cause precipitation of salts.

Before starting the first **NucleoSpin® miRNA Plasma** procedure prepare the following:

- **Wash Buffer MW2:** Add the indicated volumes of 96–100 % ethanol to the MW2 concentrate. The buffer can be stored at room temperature (18–25 °C) for at least one year.
- **RNase-free rDNase:** Add the indicated volume of Reaction Buffer for rDNase to the rDNase vial and incubate for 1 min at room temperature. Gently swirl the vial to completely dissolve the rDNase. Be careful not to mix rDNase vigorously as rDNase is sensitive to mechanical agitation. Dispense into aliquots and store at -20 °C. The frozen solution is stable for at least 6 months. Do not freeze / thaw the aliquots more than three times.

	NucleoSpin® miRNA Plasma		
	10 preps	**50 preps**	**250 preps**
REF	**740981.10**	**740981.50**	**740981.250**
Wash Buffer MW2 (Concentrate)	6 mL Add 24 mL ethanol	25 mL Add 100 mL ethanol	2 x 50 mL Add 200 mL ethanol to each bottle
RNAse-free rDNase (lyophilized)	1 vial (size C) Add 3 mL **Reaction Buffer for rDNase**	1 vial (size C) Add 3 mL **Reaction Buffer for rDNase**	5 vials (size C) Add 3 mL **Reaction Buffer for rDNase** to each vial

4 Safety instructions

The following components of the **NucleoSpin® miRNA Plasma** kits contain hazardous contents.

Wear gloves and goggles and follow the safety instructions given in this section.

GHS classification

Only harmful features need not be labeled with H and P phrases up to 125 mL or 125 g.
Mindergefährliche Eigenschaften müssen bis 125 mL oder 125 g nicht mit H- und P-Sätzen gekennzeichnet werden.

Component *Inhalt*	Hazard contents *Gefahrstoff*	GHS symbol *GHS Symbol*	Hazard phrases *H-Sätze*	Precaution phrases *P-Sätze*
rDNase, RNase-free	rDNase, lyophilized *rDNase (lyophilisiert)*	Warning *Achtung*	317-334	261, 272, 280, 304+340, 342+311, 301+312, 302+352, 333+313
MLP	Guanidinium thiocyanate 30–60% *Guanidiniumthiocyanat 30–60%*	Warning *Achtung*	302-412-EUH031	260, 273, 301+312, 330
MW1	Guanidinium thiocyanate 1–15% + ethanol 55–75% *Guanidiniumthiocyanat 1–15% + Ethanol 55–75%*	Danger *Gefahr*	225	210, 233, 403+235

Hazard phrases

H 225	Highly flammable liquid and vapour. *Flüssigkeit und Dampf leicht entzündbar.*
H 302	Harmful if swallowed. *Gesundheitsschädlich bei Verschlucken.*
H 317	May cause an allergic skin reaction. *Kann allergische Hautreaktionen verursachen.*
H 334	May cause allergy or asthma symptoms or breathing difficulties if inhaled. *Kann bei Einatmen Allergie, asthmaartige Symptome oder Atembeschwerden verursachen.*
H 412	Harmful to aquatic life with long lasting effects. *Schädlich für Wasserorganismen, mit langfristiger Wirkung.*
EUH 031	Contact with acids liberates toxic gas. *Entwickelt bei Berührung mit Säure giftige Gase.*

Precaution phrases

P 210	Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking. *Von Hitze, heißen Oberflächen, Funken, offenen Flammen sowie anderen Zündquellenarten fernhalten. Nicht rauchen.*
P 233	Keep container tightly closed. *Behälter dicht verschlossen halten.*
P 260	Do not breathe dust / fume / gas / mist / vapours / spray. *Staub / Rauc / Gas / Nebel / Dampf / Aerosol nicht einatmen.*
P 261	Avoid breathing dust. *Einatmen von Staub vermeiden.*
P 272	Contaminated work clothing should not be allowed out of the workplace. *Kontaminierte Arbeitskleidung nicht außerhalb des Arbeitsplatzes tragen.*
P 273	Avoid release to the environment. *Freisetzung in die Umwelt vermeiden.*
P 280	Wear protective gloves / eye protection. *Schutzhandschuhe / Augenschutz tragen.*
P 301+312	IF SWALLOWED: Call a POISON CENTER/ doctor/…/if you feel unwell. *BEI VERSCHLUCKEN: Bei Unwohlsein GIFTINFORMATIONSZENTRUM / Arzt /… anrufen.*
P 302+352	IF ON SKIN: Wash with plenty of water/… *BEI KONTAKT MIT DER HAUT: Mit viel Wasser/… waschen.*
P 304+340	IF INHALED: Remove victim to fresh air and keep at rest in a position comfortable for breathing. *BEI EINATMEN: An die frische Luft bringen und in einer Position ruhigstellen, die das Atmen erleichtert.*
P 330	Rinse mouth. *Mund ausspülen.*
P 333+313	If skin irritation occurs: Get medical advice / attention. *Bei Hautreizung: Ärztlichen Rat einholen / ärztliche Hilfe hinzuziehen.*
P 342+311	If experiencing respiratory symptoms: Call a POISON CENTER/ doctor/… *Bei Symptomen der Atemwege: GIFTINFORMATIONSZENTRUM /Arzt/… anrufen.*
P 363	Wash contaminated clothing before reuse. Kontaminierte Kleidung vor erneutem Tragen waschen.
P 403+235	Store in a well-ventilated place. Keep cool. *An einem gut belüfteten Ort lagern. Kühl halten.*

For further information please see Material Safety Data Sheets ***(www.mn-net.com).***
Weiterführende Informationen finden Sie in den Sicherheitsdatenblättern ***(www.mn-net.com)****.*

5 Protocol – small RNA and DNA purification from plasma or serum

Before starting with the preparation check that isopropanol is available, that ethanol was added to Wash Buffer MW2, and that rDNase was reconstituted according to section 3.

1 Prepare sample

Note: See section 2.4 for optional Proteinase K digest.

Add **90 µL Buffer MLP** to **300 µL sample**.

Vortex for **5 s**.

Incubate for **3 min** at **room temperature (18–25 °C)**.

Note: To process 600 µL or 900 µL sample material, increase volumes for Buffer MLP, MPP, and isopropanol proportionally. Multiple loading steps will be necessary in step 5. See section 2.3 for more information.

+ 90 µL MLP

Vortex 5 s

RT
3 min

2 Precipitate protein

Add **30 µL Buffer MPP** and vortex for **5 s**.

Incubate for **1 min** at **room temperature (18–25 °C).**

Centrifuge for **3 min** at **11,000 x *g*** to pellet the protein.

+ 30 µL MPP

Vortex 5 s

RT
1 min

11,000 x *g*
3 min

3 Transfer supernatant

Transfer the clear supernatant into a new Collection Tube (2 mL, lid).

Transfer supernatant

4 Adjust binding conditions

Note: Addition of carrier, for example, 2 µg of glycogen or 5 µg of LPA (linear polyacrylamide), might slightly improve the miRNA yield but usually is not necessary. Poly-A has shown only negligible effects and furthermore might interfere with photometric miRNA quantification.

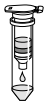
Add **400 µL isopropanol** and vortex for **5 s**.

+ 400 µL isopropanol

Vortex 5 s

5 Bind RNA and DNA

Place a **NucleoSpin® miRNA Column** in a Collection Tube (2 mL) and load the sample onto the column.



Load sample

Incubate for **2 min** at **room temperature (18–25 °C)**.

RT
2 min

Centrifuge for **30 s** at **11,000 x *g***.

11,000 x *g*
30 s

Discard the flow-through and place the column back into the collection tube.

Repeat step if necessary

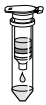
If more than 300 µL plasma / serum was used, repeat this step until all sample is loaded onto the column.

6 *Optional: DNA digest*

Note: Co-purified DNA might interfere with qPCR quantification of miRNA. The following on-column digest degrades bound DNA including miRNA genes.

However, if miRNA specific qPCR detection systems are used or cell free plasma DNA is needed intact for further analysis, skip the rDNase digest and proceed directly with step 7.

1st wash



+ 700 µL MW2

Add **700 µL Buffer MW2** to the NucleoSpin® miRNA Column. Centrifuge for **30 s** at **11,000 x *g***. Discard flow-through and place the column back into the collection tube.

11,000 x *g*
30 s

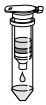
2nd wash

+ 250 µL MW2

Add **250 µL Buffer MW2** to the NucleoSpin® miRNA Column. Centrifuge for **2 min** at **11,000 x *g***. It is not necessary to discard the flow-through.

11,000 x *g*
2 min

Digest DNA



+ 50 µL rDNase

Add **50 µL rDNase** (dissolved in Reaction Buffer for rDNase according to section 3) directly onto the silica membrane of the NucleoSpin® miRNA Column.

RT
15 min

Close the lid and incubate at **room temperature (18–25 °C)** for **15 min**.

7 Wash and dry silica membrane

1st wash

Add **100 µL Buffer MW1** to the NucleoSpin® miRNA Column. Centrifuge for **30 s** at **11,000 x *g***.

+ 100 µL MW1

11,000 x *g*
30 s

Discard flow-through and place the column back into the collection tube.

2nd wash

Add **700 µL Buffer MW2** to the NucleoSpin® miRNA Column. Centrifuge for 30 s at **11,000 x *g***.

+ 700 µL MW2

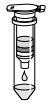
11,000 x *g*
30 s

Discard flow-through and place the column back into the collection tube.

3rd wash

Add **250 µL Buffer MW2** to the NucleoSpin® miRNA Column.

Centrifuge for **2 min** at **11,000 x *g*** to dry the membrane completely.



+ 250 µL MW2

If the liquid in the collection tube has touched the NucleoSpin® miRNA Column after the 3rd wash, discard flow-through and centrifuge again.

11,000 x *g*
2 min

Note: The same collection tube is used throughout the entire washing procedure to reduce plastic waste. If new collection tubes are to be used for each step, see ordering information.

8 Elute RNA and DNA

Place the NucleoSpin® miRNA Column in a new Collection Tube (1.5 mL).

Note: The elution buffer volume highly influences the final yield and concentration and, furthermore, influences elution efficiency of large oligonucleotides. See section 2.6 for more information about elution in 20, 30 or 50 µL.

Add **30 µL RNase-free H_2O** directly onto the silica membrane of the column.

Incubate for **1 min** at **room temperature (18–25 °C)**.

Close the lid and centrifuge for **1 min** at **11,000 x *g***.

+ 30 µL RNase-free H_2O

RT 1 min

11,000 x *g* 1 min

6 Appendix

6.1 Troubleshooting

Problem	Possible cause and suggestions
Poor or no RNA yield	*Reagents not applied or restored properly* • Always dispense exactly the buffer volumes given in the protocols! The correct proportions of buffers MLP, MPP, and isopropanol are essential for optimal yield and purity. • Always follow closely the given instructions with regard to order and mode of mixing (shaking, vortexing etc). • Add the indicated volume of 96–100 % ethanol to Buffer MW2 Concentrate and mix thoroughly. • Store kit components at room temperature (18–25 °C). Storage at lower temperatures may cause salt precipitation. Heat buffer with precipitated salt to 30 °C until salt is dissolved. Let the buffer cool down to room temperature before use. • Keep bottles tightly closed in order to prevent evaporation or contamination.
RNA is degraded	*RNase contamination* • Create an RNase-free working environment. Wear gloves during all steps of the procedure. Change gloves frequently. Use sterile, disposable polypropylene tubes and filter tips. Keep tubes closed whenever possible during the preparation unless stated otherwise. Glassware should be oven-baked for at least 2 hours at 250 °C before use.
Suboptimal performance of RNA in downstream experiments	*Inhibition by co-purified RT-PCR inhibitors* • Heme, hemin, and other degradation products of red blood cells strongly inhibit reverse transcription and PCR. Too much plasma or bad plasma quality can result in contamination with these inhibitors. Use less plasma, dilute eluates, perform the optional Proteinase K digest (see section 2.4), or add BSA prior to RT or PCR reactions.

Problem	Possible cause and suggestions
Suboptimal performance of RNA in downstream experiments *(continued)*	*Carry-over of ethanol or salt* • Do not let the flow-through touch the column outlet after the second MW2 wash. Make sure to centrifuge at the corresponding speed for the respective time in order to remove ethanolic buffer MW2 completely. • Check if buffer MW2 has been equilibrated to room temperature (18–25 °C) before use. Washing at lower temperatures lowers efficiency of salt removal. *Store isolated RNA properly* • Eluted RNA should always be kept on ice for optimal stability since trace contaminations of omnipresent RNases (general lab ware, fingerprints, dust) will degrade the isolated RNA. For short term storage freeze at -20 °C, for long term storage freeze at -70 °C.

6.2 Ordering information

Product	REF	Pack of
NucleoSpin® miRNA Plasma	740981.10/.50/.250	10/50/250 preps
Exosome Precipitation Solution (Serum / Plasma)	740398.12/.20/.60	12/20/60 mL
Exosome Precipitation Solution (Urine)	740399.12/.50/.250	12/50/250 mL
NucleoSpin® miRNA	740971.10/.50/.250	10/50/250 preps
rDNAse Set (1 vial rDNase (size D), 7 mL Reaction Buffer for rDNase)	740963	1 set
Proteinase K	740506	100 mg
Collection Tubes (2 mL)	740600	1000

Visit ***www.mn-net.com*** for more detailed product information.

6.3 Product use restriction/warranty

NucleoSpin® miRNA Plasma kit components are intended, developed, designed, and sold FOR RESEARCH PURPOSES ONLY, except, however, any other function of the product being expressly described in original MACHEREY-NAGEL product leaflets.

MACHEREY-NAGEL products are intended for GENERAL LABORATORY USE ONLY! MACHEREY-NAGEL products are suited for QUALIFIED PERSONNEL ONLY! MACHEREY-NAGEL products shall in any event only be used wearing adequate PROTECTIVE CLOTHING. For detailed information please refer to the respective Material Safety Data Sheet of the product! MACHEREY-NAGEL products shall exclusively be used in an ADEQUATE TEST ENVIRONMENT. MACHEREY-NAGEL does not assume any responsibility for damages due to improper application of our products in other fields of application. Application on the human body is STRICTLY FORBIDDEN. The respective user is liable for any and all damages resulting from such application.

DNA/RNA/PROTEIN purification products of MACHEREY-NAGEL are suitable for *IN-VITRO*-USES ONLY!

ONLY MACHEREY-NAGEL products specially labeled as IVD are also suitable for *IN-VITRO*-diagnostic use. Please pay attention to the package of the product. *IN-VITRO*-diagnostic products are expressly marked as IVD on the packaging.

IF THERE IS NO IVD SIGN, THE PRODUCT SHALL NOT BE SUITABLE FOR *IN-VITRO*-DIAGNOSTIC USE!

ALL OTHER PRODUCTS NOT LABELED AS IVD ARE NOT SUITED FOR ANY CLINICAL USE (INCLUDING, BUT NOT LIMITED TO DIAGNOSTIC, THERAPEUTIC AND/OR PROGNOSTIC USE).

No claim or representations is intended for its use to identify any specific organism or for clinical use (included, but not limited to diagnostic, prognostic, therapeutic, or blood banking). It is rather in the responsibility of the user or - in any case of resale of the products - in the responsibility of the reseller to inspect and assure the use of the DNA/RNA/protein purification products of MACHEREY-NAGEL for a well-defined and specific application.

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